Supplemental Information

Lymphotoxin beta receptor signaling limits mucosal damage through driving IL-23 production by epithelial cells

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Supplemental information includes supplementary materials and methods and 6 figures

Supplementary materials and methods

Immunohistochemistry and immunofluorescence. Detection of apoptotic cells was performed on paraffin embedded tissue sections using the Click-iT® TUNEL Alexa Fluor® 647 Imaging Assay (Invitrogen), according to the manufacturer's instructions. Proliferation of epithelial cells was measured using BrdU 3h pulse incorporation. Mice were injected i.p. with 50 μg/g BrdU (BD Biosciences) followed by anti-BrdU antibody immunohistostaining (RDI Fitzgerald). For MUC2 staining, frozen colon sections were fixed with 10% neutral buffered formalin for 10 minutes and treated with 3% (v/v) hydrogen peroxide for 30 minutes at room temperature. The sections were then incubated with PBS containing 0.3% Triton X100, 0.1% BSA, and 5% goat serum for 1h. Sections were stained overnight at 4°C with a Mucin 2 specific antibody (H300, 1:100 dilution; Santa Cruz Biotechnology Inc.) and subsequently incubated with a biotinylated anti-rabbit antibody (1:100 dilution; Vector Laboratories), followed by streptavidin HRP (1:200 dilution; eBioscience). Sections were developed with an AEC peroxidase substrate kit (Vector Laboratories), according to the manufacturer's instructions. For GFP detection, frozen colon sections were vapor fixed with 37% PFA for 1h at -20°C and blocked with 10% normal mouse serum in PBS for 30 minutes. Sections were stained for 1h with a biotin goat anti-GFP primary antibody (BA-0702, 1:100 dilution; Vector Laboratories) followed by a anti-goat-488 secondary antibody (1:100 dilution; Invitrogen) and Hoechst (1:200 dilution) for 1h.

Isolation of intestinal epithelial cells (EC). To isolate ECs, intestines were opened longitudinally, cut into 1 cm pieces and transferred to 50 ml tubes, containing 20 ml of DMEM supplemented with 5% FBS, antibiotics and 1mM DTT, incubated at 37°C with rotation (170 rpm) for 20 minutes and vortexed for 30 sec. Colon pieces were then transferred to 20 ml PBS/15mM EDTA and incubated for an additional 20 minutes with rotation (37°C). Crypts were further digested with serum free DMEM /2mg/ml Collagenase D (Roche) for 30 minutes with rotation (37°C). Single EC suspensions were passed through a 70 μm cell strainer,

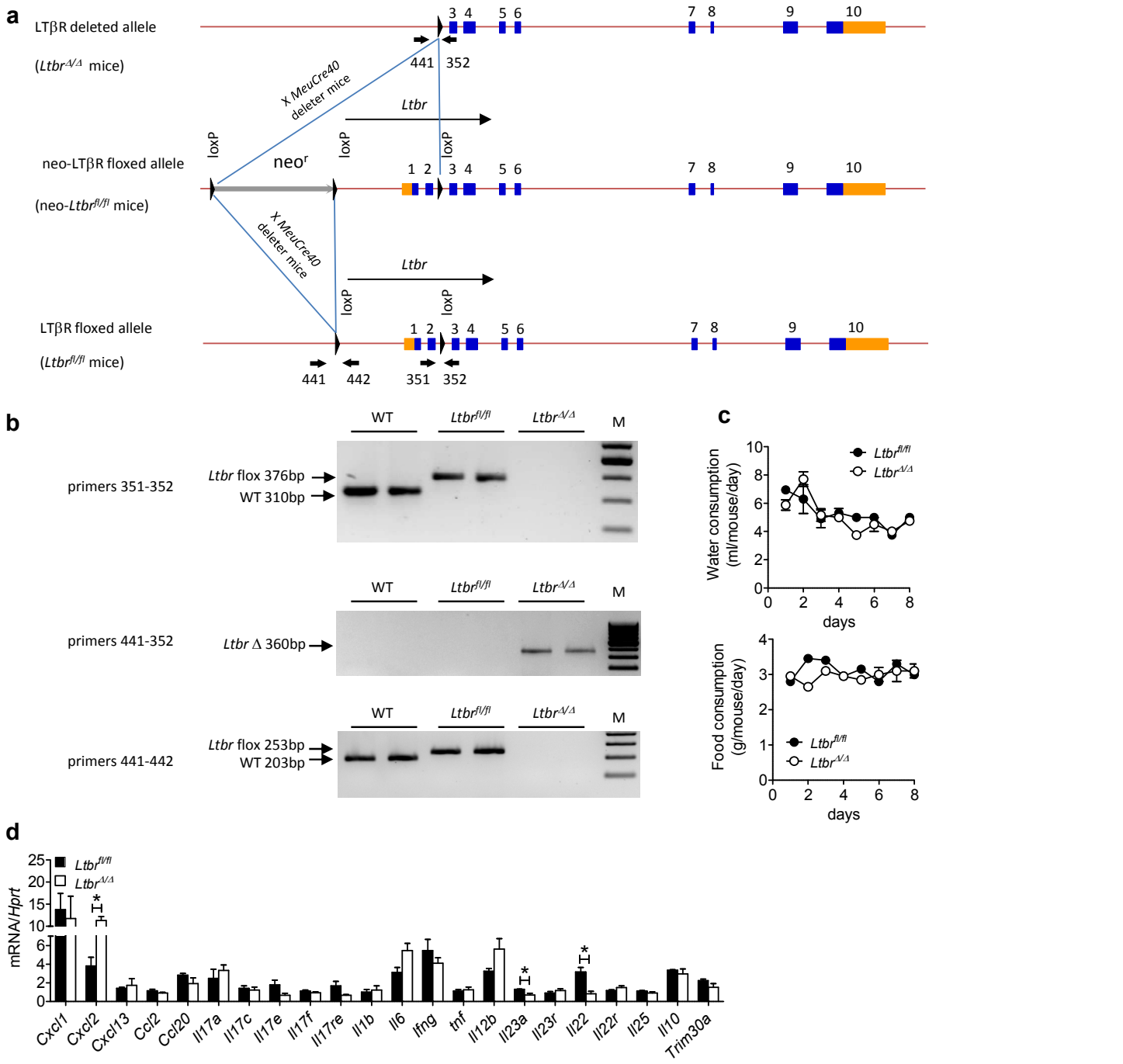
resuspended in complete media and overlaid on the top of a 20%:40% Percoll gradient. Percoll gradient separation was performed by centrifugation at 600g (1660 rpm) for 30 minutes. ECs were collected at the interphase of the 20%:40% Percoll gradient, washed and resuspended in DMEM medium.

Isolation of colonic intraepithelial lymphocytes (IELs) and *lamina propria* (LP) cells. To isolate IEL and LP cells, large intestines were opened longitudinally, briefly washed with ice-cold PBS and cut into 1 cm pieces. The tissue pieces were incubated in 20 ml of RPMI supplemented with 3% FBS, 5 mM EDTA and 0.145 mg/ml DTT for 20 min at 37°C with slow rotation. After incubation, the tissue pieces were vortexed vigorously for 30 sec and filtered using a 1mm tea strainer. The tissue pieces were then further vortexed in 10ml of 2mM EDTA/RPMI three times and all IEL containing fractions were pooled.

To isolate LP cells, the remaining tissue pieces were cut in 1 mm^2 pieces and placed in 5 ml RPMI containing 200 μg/ml Liberase (Roche) and 0.05% DNAse I (Sigma) for 20 min at 37°C with slow rotation. Digestion was performed twice. After each incubation, the solution was vortexed intensely for 20sec and passed through a 100 μm cell strainer and both LP-containing cell fractions were pooled.

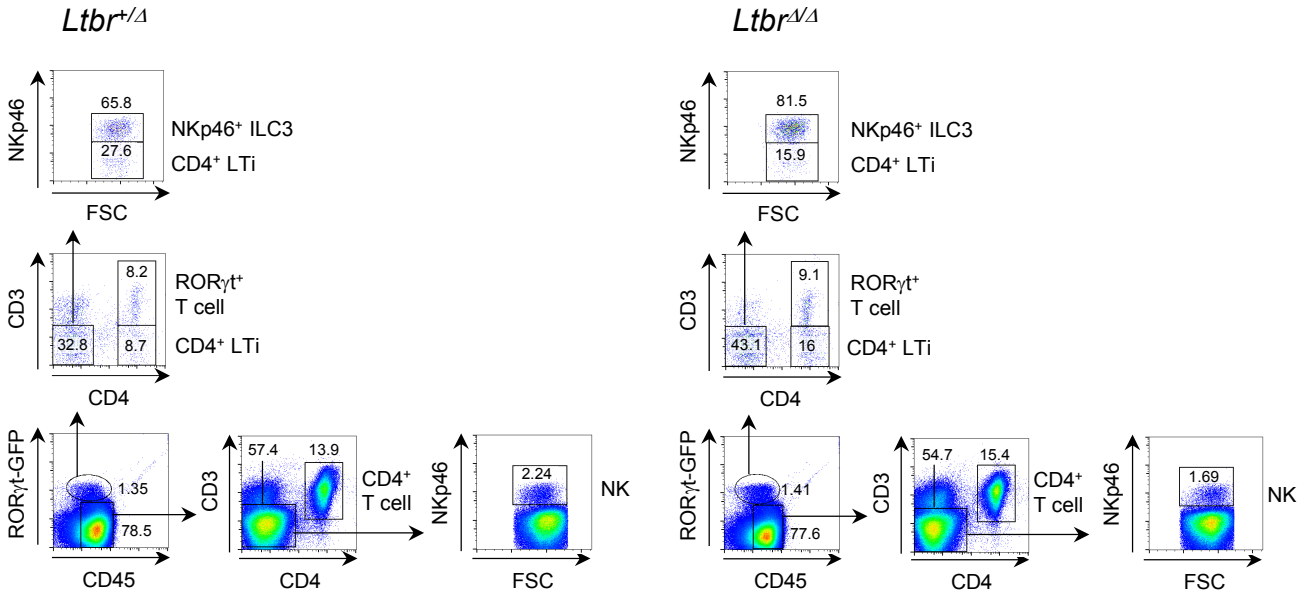
IEL and LP fractions were washed once in cold RPMI, resuspended in 40% Percoll, and overlaid on the top of 80% Percoll. Percoll gradient separation was performed by centrifugation for 20 min at 2500 rpm (1360g) at room temperature. IEL and LP cells were collected at the interphase of 40%:80% Percoll gradient, washed once, and resuspended in complete medium.

Supplementary Figure 1. Macho-Fernandez et al



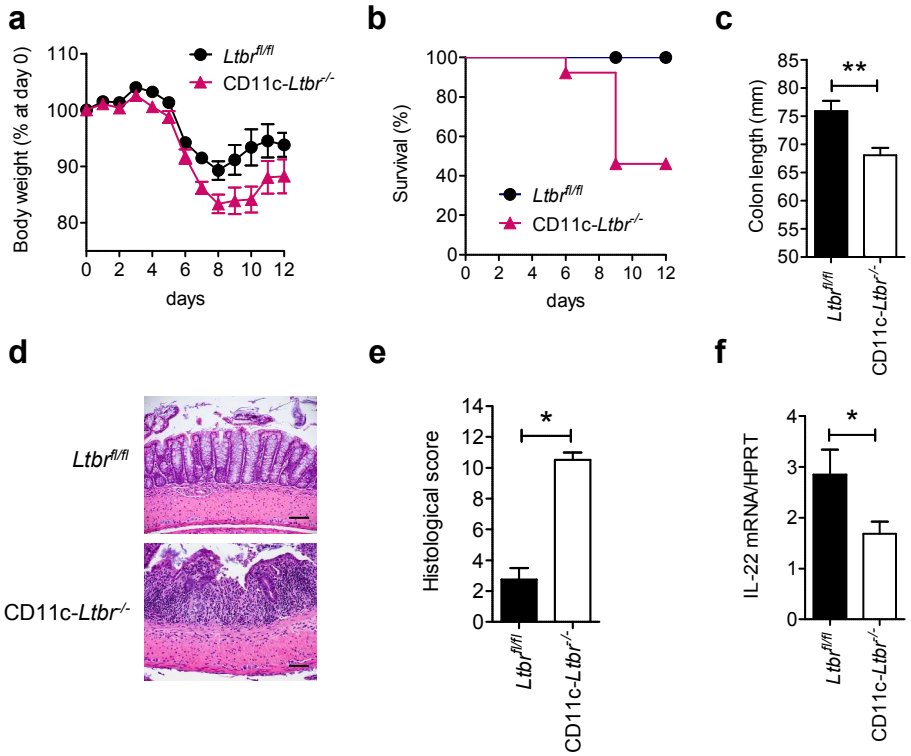
Supplementary figure 1 (**a and b**) Generation of neo-free LTβR floxed mice and neo-free LTβR-deficient mice. (**a**) Genomic locus in neo-free LTβR-deficient mice (*Ltbr*$^{\Delta/\Delta}$), neo-LTβR floxed (neo-*Ltbr*$^{fl/fl}$), and neo-free LTβR floxed (*Ltbr*$^{fl/fl}$) mice. Genotyping primers are indicated. **(b**) PCR analysis of *ltbr* gene deletion. Tail DNA from C57BL/6 (WT) mice, *Ltbr*$^{fl/fl}$ and *Ltbr*$^{\Delta/\Delta}$ mice were analyzed.
(**c**) Water and food consumption during DSS-induced colitis. *Ltbr*$^{fl/fl}$ and *Ltbr*$^{\Delta/\Delta}$ mice were treated for 5 days with 3.5% DSS. Water and food consumption were evaluated daily.
(**d**) Expression of genes associated with promotion or regulation of intestinal inflammation. Expression of genes was evaluated in the colon of *Ltbr*$^{fl/fl}$ and *Ltbr*$^{\Delta/\Delta}$ mice treated for 5 days with 3.5% DSS (n=5). Data are normalized to *Hprt*. **P*<0.05 (Student's *t*-test). Data are representative of three independent experiments. Error bars represent SEM.

Supplementary Figure 2. Macho-Fernandez et al



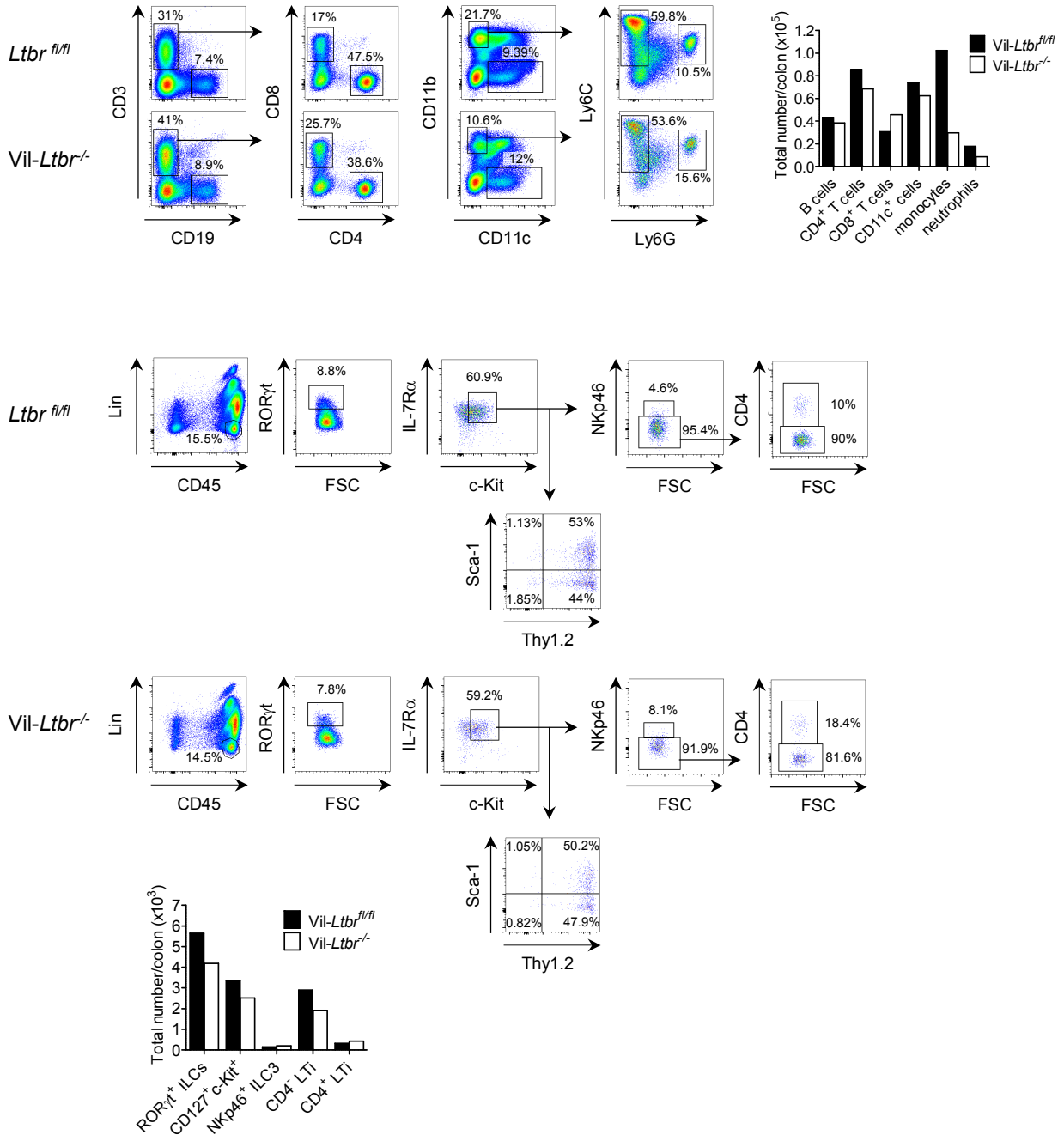
Supplementary Figure 2 Sorting strategy for purification of colonic *lamina propria* cell populations. *Lamina propria* (LP) cells were purified from the colon of RORγt-GFP$^{+/-}$ *Ltbr*$^{+/\Delta}$ and RORγt-GFP$^{+/-}$ *Ltbr*$^{\Delta/\Delta}$ reporter mice treated with 3.5% DSS for 5 days (n=3). Sorted LP cell populations are indicated as: CD4^{-} LTi (CD45^{+}RORγt^{+}CD3^{-}CD4^{-}NKp46^{-}), CD4^{+} LTi (CD45^{+}RORγt^{+}CD3^{-}CD4^{+}NKp46^{-}), NKp46^{+} ILC3 (CD45^{+}RORγt^{+}CD3^{-}CD4^{-}NKp46^{+}), RORγt^{+} T cell (CD45^{+}RORγt^{+}CD3^{+}CD4^{+}NKp46^{-}), CD4^{+} T cell (CD45^{+}RORγt^{-}CD3^{+}CD4^{+}NKp46^{-}) and NK cell (CD45^{+}RORγt^{-}CD3^{-}CD4^{-}NKp46^{+}).

Supplementary Figure 3. Macho-Fernandez et al



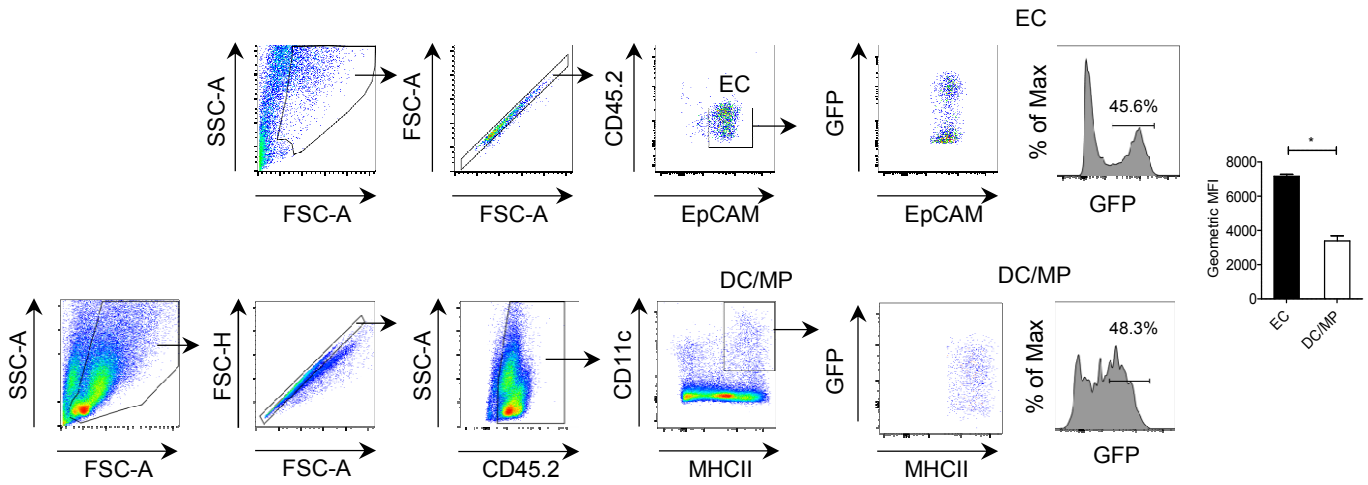
Supplementary Figure 3. LTβR signaling in CD11c^{+} cells contributes to protection against DSS-induced injury. *Ltbr*$^{fl/fl}$ and CD11c-*Ltbr*$^{-/-}$ mice (n=5) were treated for 5 days with 3.5% DSS.
(**a**) Weight loss and (**b**) survival. (**c**) Colon length, and (**d**) hematoxylin and eosin staining and histological score (**e**) of colon sections at day 11. (**f**) Colonic expression of *Il22* mRNA was evaluated at day 5. For mRNA expression, data are normalized to *Hprt*. **P*<0.05; ***P*<0.01 (Student's *t*-test). Data are representative of two independent experiments. Error bars represent SEM.

Supplementary Figure 4. Macho-Fernandez et al



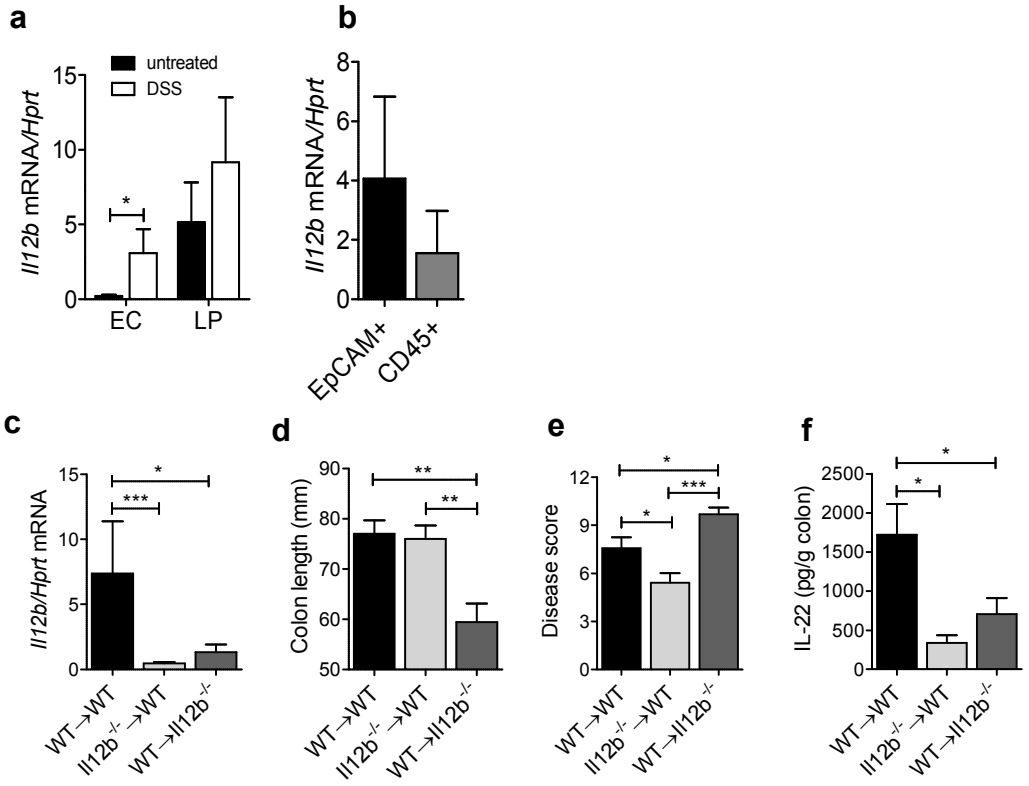
Supplementary Figure 4. Vil-*Ltbr*$^{-/-}$ mice display a normal recruitment of innate and adaptive immune cells to the colonic *lamina propria* during DSS-induced colitis. Colonic LP cells from *Ltbr*$^{fl/fl}$ mice and Vil-*Ltbr*$^{-/-}$ mice treated for 5 days with 3.5% DSS (n=3 per group) were analyzed by flow cytometry.

Supplementary Figure 5. Macho-Fernandez et al

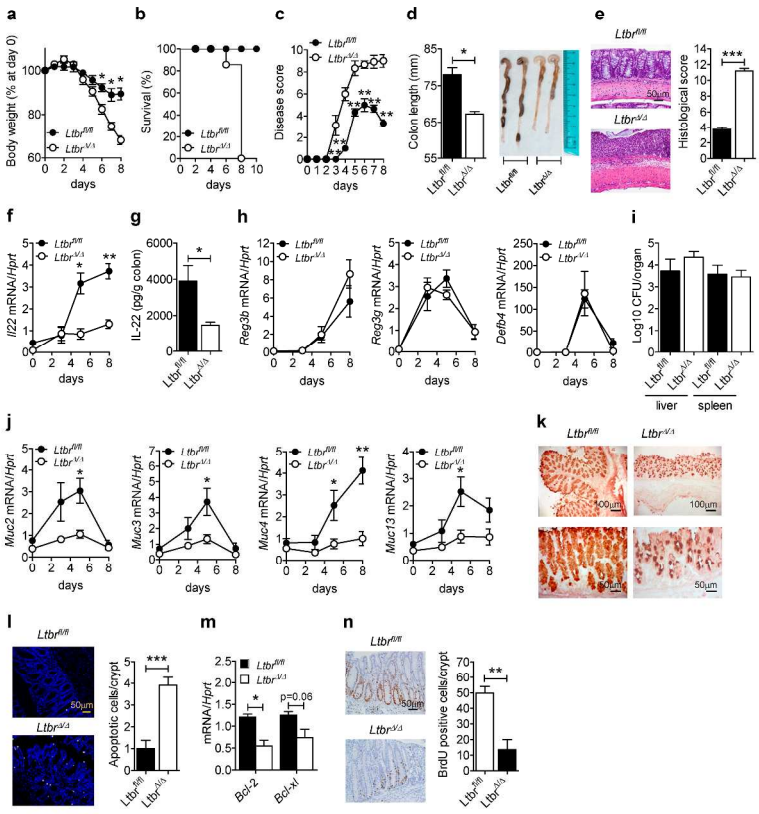


Supplementary Figure 5. Epithelial cells produce IL-23 in response to mucosal damage.
IL-23a-GFP$^{+/-}$ mice were treated with 3.5% DSS for 5 days (n=3). IL-23a-driven GFP expression in purified epithelial cells (EC, top panels) and dendritic cells and macrophages (DC/MP, bottom panels) was evaluated at day 5 in the colon of IL-23a-GFP$^{+/-}$ mice by flow cytometry. **P*<0.05 (Student's *t*-test). Error bars represent SEM.

Supplementary Figure 6. Macho-Fernandez et al



Supplementary Figure 6. Epithelial cell-derived Il12b promotes mucosal healing.
(**a** and **b**) Epithelial cells express Il12b in response to mucosal damage. WT (C57Black/6) mice were treated with 3.5% DSS for 5 days or left untreated (n=3 mice per group). (**a**) *Il12b* mRNA expression in percoll-purified fractions of epithelial cells (EC) or lamina propria (LP) cells from colon. (**b**) *Il12b* mRNA expression in sorted $EpCAM^{+}CD45^{-}$ or $EpCAM^{-}CD45^{+}$ cells from percoll-purified EC fraction from colon.
(**c-f**) Expression of Il12b in radioresistant cells promotes mucosal healing. Bone marrow cells from C57BL/6 (WT) or *Il12b*$^{-/-}$ mice were transferred into lethally-irradiated WT or *Il12b*$^{-/-}$ mice. Six weeks later, WT→WT, *Il12b*$^{-/-}$ →WT, and WT→ *Il12b*$^{-/-}$ chimeric mice were treated for 5 days with 5% DSS.
(**c**) *Il12b* mRNA expression in the colon of chimeric mice at day 8.
(**d-e**) Colon length and clinical disease score at day 8.
(**f**) IL-22 production in the supernatants of colons collected at day 8 and cultured for 24h. For mRNA expression, data are normalized to *Hprt*. **P*<0.05; ***P*<0.01; ****P*<0.001 (Student's *t*-test). Data are representative of two independent experiments with 3-8 mice per group. Error bars represent SEM.



Macho-Fernandez et al Figure 1

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